Submission by Philip Morris U.S.A.

to

The National Toxicology Program

- Appendix 6 -

Measurement of Cotinine Concentration in Biological Fluids and Use of the Results in Epidemiological and Exposure Studies

March 20, 1998

2063656412

Source: https://www.industrydocuments.ucsf.edu/docs/qfpk0000

Introduction

There are two reasons that a biomarker for tobacco use or environmental tobacco smoke (ETS) exposure is of considerable interest. The first is to determine whether an individual is a smoker or nonsmoker. The second is to attempt to quantify levels of exposure to ETS for nonsmokers. To answer the first question is relatively straightforward, since in principle the difference between a given biomarker for a smoker and a nonsmoker should be large. The second question, however, requires one to attempt to differentiate between two small and similar values. As a result, the analytical criteria for these two applications are very different, and require consideration of different methodologies. In addition to the analytical concerns, the potential impact of interpersonal variation in metabolic rates and pathways contributes confusion. Add the choice of saliva, serum, or urine as the sample matrix, and it is easy to see why a complex set of measurement and data interpretation uncertainties has evolved. Some of these issues will be addressed in this discussion.

Cotinine is a carbon-oxidation metabolite of nicotine. It is found at measurable levels in saliva, serum and urine of subjects who smoke, who use other forms of tobacco, or who have been exposed to ETS at sufficient levels (Benowitz, 1996). Cotinine meets many of the criteria proposed by the National Research Council (NRC) for an ETS biomarker (NRC, 1986), but it must be kept in mind that cotinine is derived from nicotine. Although cotinine may serve as a biomarker for nicotine intake, it may not serve as well as a biomarker for ETS exposure. Furthermore, cotinine has a biological half-life of approximately 20 hours and can only represent nicotine intake within an

2063656413

Source: https://www.industrydocuments.ucsf.edu/docs/qfpk0000

interval of a few half-lives. While a number of alternative biomarkers have been proposed, most of these have been devaluated relative to cotinine (Benowitz, 1996, Table 5). In the search for a biomarker for tobacco use or ETS exposure, by a process of elimination cotinine appears well on the way to becoming the compound of choice for many researchers (Benowitz, 1996).

Analytical methods may be described as selective or as specific. Although many analytical methods exist for specific detection and quantification of cotinine, most of these are based on instrumental methods of analysis (Benowitz, 1996, Table 4). Frequently, these methods are considered too expensive, too labor intensive, or to require special skills beyond those frequently available for epidemiological or exposure studies. Radioimmunoassay (RIA) methods for cotinine were developed and widely applied in the scientific community that conducts various epidemiological and exposure investigations (Langone et al., 1973). RIA methods are rapid, economical and require few special analytical skills, but they are selective rather than specific for chemical compounds.

Selectivity was likely thought to be an adequate criterion for cotinine. Until approximately 1987, cotinine was considered to be the major, if not the only, known nicotine metabolite of analytically significant concentration in the biological fluids of interest. Although N-oxidation metabolites of nicotine were known, they were found only at low concentrations (Turner, 1969). After 1987, the presence of significant concentrations of *trans*-3-hydroxycotinine (Neurath and Pein, 1987), and glucuronide conjugates of nicotine, cotinine and *trans*-3-hydroxycotinine (Curvall et al., 1989) in urine were recognized. Although cotinine appears to be the major nicotine

2063656414

Source: https://www.industrydocuments.ucsf.edu/docs/qfpk0000

metabolite in saliva and serum (Curvall et al., 1990), *trans*-3-hydroxycotinine (Byrd et al., 1994) or its glucuronide conjugate (Andersson et al., 1997) is the most prevalent in urine. Actually, cotinine constitutes only 10-15% of the nicotine metabolites. At least seventeen (17) metabolites of nicotine have been identified in urine (Kyerematen et al., 1987). Many of these have been quantified (Byrd et al., 1994; Andersson et al., 1997).

Cotinine as a Biomarker for Smoker Classification

The evolution of cotinine as a biomarker began as a means to discern smokers from nonsmokers. The levels of cotinine in saliva, serum and urine of a smoker of >20 cigarettes per day is relatively easy to distinguish from that of a nonsmoker using a variety of analytical methods including RIA. In large epidemiological studies for which cost, labor, time and other issues are important, saliva samples are the easiest to obtain. It has been reported that analytical determinations of cotinine in saliva provide essentially the same information as similar determinations in serum (Curvall et al., 1990). A mean saliva to serum ratio of cotinine concentration of 1.2 has been reported (Curvall et al., 1990). Although further study is needed, at this time both matrices are thought to be relatively free of other analytically significant metabolites of nicotine. The principal reason that nicotine is not used as a biomarker for its uptake is that the half-life of nicotine in biological fluids is only approximately 2 hours (Benowitz, 1996). Measurement of nicotine would not give a representative estimate of nicotine intake during the previous few days.

2063656415

Source: https://www.industrydocuments.ucsf.edu/docs/qfpk0000

In epidemiological or exposure studies, classification of an individual as a smoker or nonsmoker is critical to the results, especially in the interpretation of data at low relative risk (RR) levels such as those reported with regard to ETS exposure (Jenkins et al., 1996). Wide ranges of smoking status misclassification have been suggested based on questionnaire data (Riboli et al., 1995), and misclassification of 2 -5% smokers claiming to be nonsmokers is not uncommon (Jenkins et al., 1996; Phillips et al., 1996). RR values below approximately 2.0 are considered weak associations (Greenberg, 1986). In the area of ETS epidemiology, low RR values, as for example 1.2, could be significantly influenced by smoking status misclassification of less than 5%.

Utilization of cotinine as a biomarker for smoker classification is less of an analytical issue than a policy issue. Interpersonal variability in metabolic processes, lifestyle, and other factors creates variations in cotinine levels that make it very difficult to distinguish an occasional smoker from a nonsmoker with recent exposure to high levels of ETS (Phillips et al., 1996). An additional issue that is rarely addressed in the literature is "intra-personal" variability. For example, researchers who conduct exposure studies infrequently collect more than one, possibly two, samples from an individual subject. Ranges of exposure that result from daily lifestyle variations are not considered. This variability may well be much greater than the analytical variability.

As a result, uncertainty exists over the cotinine concentration that unambiguously differentiates a smoker from a nonsmoker. At the present time, somewhat empirical decisions are made. These decisions may be influenced by the goal of the investigation. It has been suggested that cotinine concentrations of 10-50 ng/mL in saliva or serum, or 50 nanogram cotinine per milligram

2063656416

Source: https://www.industrydocuments.ucsf.edu/docs/qfpk0000

creatinine in urine (cotinine-creatinine ratio: CCR) are levels below which a subject is considered to be a nonsmoker and above which is classified as a smoker. Conflicts with questionnaire data arise and are occasionally resolved by dismissing the subject from the study. The latter decision is prudent for experimental purity, but could eliminate an important segment of the total population.

The complex distribution of cotinine species in urine would recommend the use of serum or saliva for smoking status classification. Pirkle et al. (1996) have determined serum cotinine levels for over 4,000 subjects spanning wide age groups, smoking status and ETS exposure. Their results show a finite percentage of the population with serum cotinine levels below 10 ng/mL that report tobacco use, and above 10 ng/mL that report no tobacco use or ETS exposure at home or at work. Three regions of classification should be considered: smokers, nonsmokers and indeterminate. The overlap is a combined result of interpersonal metabolic variances, variation in the degree of ETS exposure in nonsmokers, inaccurate questionnaire-derived data and analytical variance. These levels of uncertainty not only have potential for impact on conclusions drawn from scientific studies, but on decisions regarding individuals such as classification for other purposes.

In summary, when an individual is clearly a smoker, or clearly a nonsmoker with moderate ETS exposure, cotinine measurements in saliva, serum or urine may be capable or distinguishing between the two. To determine the classification of occasional smokers or nonsmokers with significant ETS exposure is not an analytical issue, but one of establishing a decision point. Establishing this point is complicated by the lack of a clear definition of a smoker. If the cotinine level used to classify a subject as a smoker is too high, smokers may be erroneously

2063656417

Source: https://www.industrydocuments.ucsf.edu/docs/qfpk0000

classified as nonsmokers. One study (Saracci and Riboli, 1989) that classified as smokers subjects with urinary levels of cotinine above 50 ng/mg creatinine, later reported that of the 47 subjects that were excluded from the study as smokers, only 20 (1.5% of the total study population) can be considered to be smokers (Riboli et al., 1995). The authors suggested that a cutoff level of 150 ng/mg should be used to avoid exclusion of nonsmokers with high ETS exposure. The position could be taken that a level lower than 50 ng/mg is more realistic to identify light smokers who are deceivers in questionnaire responses. This example clearly demonstrates that a single point value used for all subjects is woefully inadequate for such an important decision. Selection of the point value criteria may be driven inadvertently by the goal of the study.

Cotinine as a Biomarker for ETS Exposure

The analytical chemistry issues for the use of cotinine as a biomarker for classification of smoking status are minor compared to those associated with its use for estimating ETS exposure-dose relationships. The evolution of the use of cotinine levels from a method to make a binary decision (smoker or nonsmoker) to implied quantification of ETS exposure was a slow but relentless process. Cotinine concentrations in the case of regular smokers are normally 2-3 orders of magnitude greater than of the limit of quantification (LOQ) of analytical methods that have been applied. Even the marginal region of 1-5 ng/mL in saliva, serum and urine are adequately measurable using RIA and chromatographic methods.

2063656418

Source: https://www.industrydocuments.ucsf.edu/docs/qfpk0000

Especially in urine samples, analytical and interpersonal metabolic considerations may become paramount. These factors, coupled with what may be considered a propensity toward analytical expediency in epidemiological ETS exposure studies, may lead to opportunities for contradictory conclusions. Recently, prominent professors of epidemiology expressed the view that contradictory findings in epidemiological research are "common" (statements of Trichopoulos et al. 1997). It is possible that some of these contradictions stem from inadequate experimental design, insufficient number of subjects, or from errors in the analytical data upon which decisions are based.

As mentioned above, saliva, serum and urine have all been used as matrices for the determination of nicotine and its metabolites. Because urine has been used in a large number of epidemiological studies, and because that matrix appears to contain the largest number of metabolites, it will be considered first.

Metabolic Products of Nicotine in Urine

At least 17 metabolic products of nicotine have been identified in human urine (Kyerematen et al., 1987). Of these, cotinine has been the focal component. Analytical methodologies have been developed that may be applied reasonably to those compounds shown in **Table 1**, along with some of their glucuronide conjugates. The remaining metabolites require more involved analytical methods. **Table 2** shows the results of two independent studies that measured in human urine the compounds shown in **Table 1**. The results are reported as percent of the total found based on nicotine equivalents of each compound, and the cumulative total if the compounds

2063656419

Source: https://www.industrydocuments.ucsf.edu/docs/qfpk0000

Table 1. Nicotine and metabolites that have been determined analytically.

Compound	Abbreviation
Nicotine	NIC
Nicotine glucuronide conjugate	NIC-G
Cotinine	COT
Cotinine glucuronide conjugate	COT-G
trans-3'-Hydroxycotinine	3HC
trans-3'-hydroxycotinine glucuronide conjugate	3HC-G
Nicotine-N'-oxide	NNO
Cotinine-N-oxide	CNO
Demethynicotine	DMC

2063656420

Source: https://www.industrydocuments.ucsf.edu/docs/qfpk0000

Table 2. **Distribution of nicotine and metabolites in two studies.**
(Each compound was converted to an equivalent amount of nicotine.)

NIC + metabolites	% of total found[1]	cumulative % total	% of total found[2]	cumulative % total
3HC	35	35	36	36
COT-G	17	52	14	50
COT	13	65	9	59
NIC	10	75	9	68
3HC-G	9	84	23	91
NIC-G	3	87	5	96
NNO	7	94	3	99
CNO	4	98	1	100
DMC	2	100	Not Determined	
(Smokers) N =	11		91	
PERCENT N-OXIDE	11		4	

1. Byrd et al. (1994) (SD values are 3-8%, taken from Table I, RSD values calculated to be 21-75%).

2. Andersson et al. (1997) (SD values are 0.9-10.6 taken from Table 4, RSD values calculated to be 28-100%).

2063656421

Source: https://www.industrydocuments.ucsf.edu/docs/qfpk0000

are taken into account sequentially. There is general agreement between these mean values reported in the two studies, although a significant difference was found between the distribution of the glucuronide conjugates, especially the 3HC-G. The variance of the mean of the percentage of each metabolite is somewhat higher in the study by Andersson et al. (1997) than in the one by Byrd et al. (1994).

It is clear from **Table 2** that free (i.e., not conjugated) cotinine accounts for only about 10% of the total nicotine metabolites analytically detectable in human urine. This observation requires a number of considerations:

1. Whether a selective (i.e., RIA) or specific (i.e., GC/MS) analytical method is used for cotinine determination, only about 10% of the nicotine equivalent as cotinine will be measured unless an enzyme-catalyzed hydrolysis is conducted to free the conjugated cotinine. Even then only a portion of the nicotine metabolites is measured.

2. In the case of RIA, a cross reactivity of approximately 34% for *trans*-3'-hydroxycotinine (3-HC) in urine samples has been reported (Zuccaro et al., 1997). Multiple regression of data presented by Zuccaro et al. (1997) yields an expression for cotinine concentration determined by RIA ($[COT]_{RIA}$) versus cotinine concentration ([COT]) and trans-3'-hydroxycotinine ([3-HC]) determined by HPLC as follows (concentrations in μg/L):

$$[COT]_{RIA} = 0.97\pm(0.63)\,[COT] + 0.29(\pm0.14)[3\text{-}HC] + 557(\pm371)$$

2063656422

Source: https://www.industrydocuments.ucsf.edu/docs/qfpk0000

Interestingly, both the coefficient of variation and the "p" value for the coefficient for 3-HC are smaller than that for COT. This suggests that the results from RIA determinations as used by Zuccaro et al. are more strongly correlated to the [3-HC] than to [COT], although more selective for cotinine.

The results of both studies represented in **Table 2** indicate that the mean *trans*-3'-hydroxycotinine is about three times the concentration of cotinine in urine. RIA would yield a result that is a combination of cotinine and cross reactivity with *trans*-3'-hydroxycotinine. The analytical bias can be as high as a factor of two, and leads to considerable confusion when RIA results are compared to GC or GC/MS results.

3. If enzymatic hydrolysis of the conjugates is included in the analytical step, the resulting nicotine, cotinine and *trans*-3'-hydroxycotinine concentrations determined by chromatography could account for approximately 85-90% of the total nicotine and its metabolites found in urine samples. This can be concluded from the cumulative total columns in **Table 2**. The only reliable approach to relate nicotine dose (and, thereby, uptake) to nicotine metabolites in urine for individuals is to perform a complete suite of metabolite determinations to account for pharmacokinetic differences.

4. The results in **Table 2** represent mean values that are reasonably consistent between the two studies. However, the variation of each mean can be up to 100%. Some of this distribution is analytical error; some of it is interpersonal variation. Although correlation between means

2063656423

Source: https://www.industrydocuments.ucsf.edu/docs/qfpk0000

in different matrices has been established (Benowitz, 1996), no controlled experiments are known to determine the variance between individual values either on an interpersonal or intrapersonal level.

In summary, with frequently used analytical approaches for determination of cotinine in urine, only about 10% of the total nicotine is accounted for in the analysis. When RIA is used, the representation as a cotinine determination may be in error by as much as a factor of two. Approximately 25-35% of the metabolites is present as conjugates not detected unless enzymatic hydrolysis is conducted. Interpersonal variation is an important issue dealing not only with nicotine exposure, but also with metabolic rates and distribution among the various metabolites and their conjugates. When only 10% of the metabolite products of nicotine are used, it is not unexpected that large variations in results will be obtained.

Finally, urine volume is a variable associated with many factors including liquid consumption. It is possible that careful management of 24-hour urine sampling can be used successfully (Benowitz et al., 1997). Unfortunately, many studies related to ETS exposure make determinations using only one sample. Use of the cotinine-creatinine ratio (CCR) to adjust for urine output has limitations because of the variables that influence creatinine output.

2063656424

Source: https://www.industrydocuments.ucsf.edu/docs/qfpk0000

Metabolic Products of Nicotine in Serum and Saliva

Little has been reported in the literature concerning nicotine metabolites other than cotinine in blood. From the point of view of analytical significance, cotinine appears to be the most important nicotine metabolite in serum. The conversion of nicotine to cotinine in blood by the liver of smokers has been related to daily nicotine intake as 0.08 [(mg/24 hr)/ng/mL], with a coefficient of variation of 21.9% (Benner et al., 1989) Thus, a serum cotinine level of 250 ng/mL corresponds to a daily intake of 20±4 mg of nicotine. It was suggested that this factor also applies to nonsmokers (Benowitz, 1996).

Benowitz (1996) has described a calculation that suggests a urine to blood ratio of 6, but cautions that the interpersonal variability described previously would contribute to variability in this ratio. There are insufficient data to estimate the interpersonal variability. The ratio predicted by Benowitz has had some experimental verification with a urine to blood cotinine ratio of 5 reported (Jarvis et al., 1984). Benowitz (1996) also reports that saliva to blood ratios are 1.1-1.4, leading to an essentially interchangeable use of saliva and serum cotinine data. These conversions have been summarized in **Table 3**.

Using the serum to daily nicotine intake conversion given above, and data from a large study (Pirkle et al., 1996, Figure 2), the approximate daily intake for nonsmoking subjects with no reported ETS exposure, reported ETS exposure and reported smokers is 0.024, 0.08, and 40 mg, respectively. Using a similar approach to a different set of data, a group of 91 smokers with mean

2063656425

Source: https://www.industrydocuments.ucsf.edu/docs/qfpk0000

Table 3. Conversion factors for cotinine.

To convert	Multiply by
Cotinine in serum to cotinine in urine ng/mL → ng/mL	5-6
Cotinine in serum to cotinine in saliva ng/mL → ng/mL	1.1-1.4
Cotinine in serum to daily (24 hr) nicotine intake ng/mL → mg/day	0.08±0.18

2063656426

Source: https://www.industrydocuments.ucsf.edu/docs/qfpk0000

saliva cotinine concentrations of 261.1 (±116.2) ng/mL would convert to a daily intake of 20.9 (±3.8) mg of nicotine, which may be compared to the experimentally determined 19.8 (±6.3) (Andersson et al., 1997). Other examples reinforce the evidence that serum or saliva cotinine levels can be used to estimate the mean values of nicotine intake, but the variance about those estimates for individuals may be significant, as described below. Furthermore, most of the available data are based on single samples from each individual, and very little is know about intra-individual variation.

Use of Cotinine as a Nicotine Biomarker in Exposure Studies

In a large study by Jenkins et al. (1996), saliva samples for cotinine measurements were taken for nonsmoker subjects in 16 cities in the United States the day before and the day after personal monitors were used to measure ETS parameters, including nicotine, in workplace and non-workplace environments. Four cell types were defined depending on smoking taking place away from work or in the workplace. A strong correlation ($R^2 = 0.991$) was found between the median 24-hr time weighted average (TWA) nicotine exposure and the median average salivary cotinine level on a cell-by-cell basis. Salivary cotinine levels were not well correlated with individual 24-hr TWA nicotine levels (R^2=0.105). Based on an earlier study discussed above (Saracci and Riboli, 1989), nonsmokers not exposed to ETS had an apparent estimated daily intake of 0.024 mg of nicotine, and nonsmokers exposed to ETS 0.08 mg. These compare favorably with similar calculations using the data of the study by Jenkins et al. (1996). For the cell with nonsmoking home and nonsmoking workplace, the calculated daily intake is 0.013 mg (vs. 0.024), whereas, for the subjects in the cell with smoking away from work but not at work, an estimated daily intake of 0.07

2063656427

Source: https://www.industrydocuments.ucsf.edu/docs/qfpk0000

mg (vs. 0.08) is calculated. In the most extreme case of ETS exposure both at work and away from work, a daily nicotine intake of 0.16 mg is estimated. These results appear to be consistent findings, although the Jenkins data were less so when salivary cotinine levels were below 2 ng/mL. All comparisons are based on mean or median values and may not be valid for individuals.

Although salivary cotinine measurements have been used as a biomarker for nicotine exposure for means (or medians) of large numbers of subjects from within given exposure groups, a wide range of levels of salivary cotinine is found for individual smokers and nonsmokers alike that does not correlate well to their nicotine exposure levels (Saracci and Riboli, 1989). Because of the consistent relationships between cotinine in saliva, serum and urine, it is concluded that none of these matrices can be reliably applied to individual subjects using a single measurement. As stated above, analytically valid measurements of cotinine in any of these matrices can distinguish a smoker of 20 cigarettes per day from a nonsmoker who is not exposed to ETS. Distinction between a light smoker and a nonsmoker exposed to high ETS levels is very problematic. Kemmeren et al. (1994) have used a statistical equation shown below, and based on expressions for "t" values, to predict the number of replicate measurements needed to estimate the "habitual" serum cotinine level of a subject within a selected percentage of the "true value." The expression used is

$$k_{0.95} = (1.96 \times CV/D)^2$$

in which "k" is the number of measurements needed to estimate the habitual cotinine level within a certain percentage "D" of the intra-personal variation "CV" at a 95% confidence level. The intra-

2063656428

Source: https://www.industrydocuments.ucsf.edu/docs/qfpk0000

personal coefficient of variation (CV) was estimated to be 16.1%, although there is little supporting evidence for this value. Using this value, one calculates that a single sample can estimate the "habitual" cotinine concentration to within approximately ±32%. Ten (10) replicate samplings would be required to obtain a measure within ±10% of the "habitual" value. The issue of the time period over which sampling and measurements were or should be made was discussed briefly by Kemmeren et al. (1994), who observed that samples should be taken over a longer period of time than just a few days.

In summary, it does not seem appropriate to debate a point value for a cutoff to distinguish smokers from nonsmokers based on a single measurement of salivary cotinine. In this case, the intra-personal variation in the concentration will contribute significantly to the cotinine level, and less is known about the interpersonal variation with a fixed uptake of nicotine. These pharmacokinetic and lifestyle factors must be given more consideration when attempting to use a point value for decision making.

Salivary Cotinine Concentration Related to Air Quality Measurements

Benowitz (1996) has discussed relationships between cotinine measurements and nicotine intake. The relationships proposed have been described earlier and provide some opportunity to assess nicotine intake on a daily average. Three ETS exposure studies involving subjects from 16 cities in the United States (Jenkins et al., 1996), Stockholm, which has exceptionally low ETS (nicotine) levels (Phillips et al., 1996), and Barcelona, which tends to have

2063656429

Source: https://www.industrydocuments.ucsf.edu/docs/qfpk0000

relatively high levels of ETS both in the workplace and at home (Phillips et al., 1997), provide an opportunity to consider such relationships. In all three studies, salivary cotinine measurements were made using the RIA method both prior to and after the subjects' breathing zones were sampled using personal monitors. In the case of working subjects, dual personal monitors were used for workplace and away from workplace environments. The data obtained from the monitor samples were used to calculate 24-hour time weighted average (TWA) nicotine concentrations.

Figure 1 shows a plot of the median salivary cotinine concentration versus the 24-hr TWA nicotine in air concentration for different subject categories for all three studies. The median was used because at very low levels near analytical detection limits it is generally a more representative value than the mean. In most cases, at higher concentrations the mean and median were relatively close in value. One clear outlier point is observed in the results shown in **Figure 1**. This point represents non-working housewives or househusbands living in a nonsmoking home environment in Stockholm (Phillips et al., 1996). That datum was not included in the regression. No explanation was provided for this result, which represents by far the highest nicotine and cotinine levels found in the Stockholm study, and is almost 10-fold higher than that found for homes of workers with smoking environments. Only nine (9) subjects were in this group, and it is possible that the values obtained were not representative. There could also have been unknown extraneous sources of nicotine.

After removing the datum discussed above, the remaining data shown in **Figure 1** yield the following linear regression.

2063656430

Source: https://www.industrydocuments.ucsf.edu/docs/qfpk0000

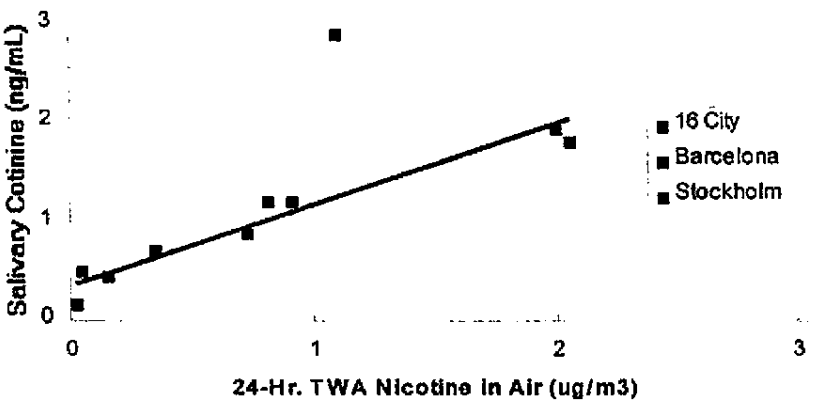

Figure 1. Plot of Salivary Cotinine Concentration versus 24-hr. TWA Nicotine Concentration Exposure in Nonsmokers

2063656431

Source: https://www.industrydocuments.ucsf.edu/docs/qfpk0000

Salivary Cotinine (ng/mL) = 0.76(±0.07)*Nicotine ($\mu g/m^3$) + 0.38(±0.07)

For both the coefficient and intercept, $p<0.001$. However, again it should be emphasized that this correlation is for median values of groups of subjects ranging from as few as 9 to over 100. Individual variation is expected to be large, but the data were not made available in the publications. Furthermore, geographic location appears to play a significant role (Jenkins et al., 1996; Phillips et al., 1996; Phillips et al., 1997). Thus, there is no evidence that salivary cotinine concentration can be used to estimate ETS exposure for an individual using such a correlation.

The intercept in the regression of the data shown in **Figure 1** is different from zero with statistical significance. The implication of this intercept is that the mean salivary nicotine concentration in all groups has a base level that is not derived from nicotine in ETS. Using the factor shown in **Table 3**, a daily intake of 30 μg/24 hr from an additional source is estimated. It is tempting to suggest that there are sources of cotinine in saliva of nonsmokers other than that from ETS exposure. Dietary sources have been suggested (Castro and Monji, 1986; Sheen, 1988; Davis et al., 1991; Domino et al., 1993; Domino, 1995). There are still open questions concerning potential cross-reactivity and other issues related to the RIA method of analysis, so that the potential for an artifact cannot be ruled out.

A factor that contributes to the intercept described above is the data analysis policy used. In the indoor air studies mentioned above (Jenkins et al., 1996; Phillips et al., 1996; Phillips et al., 1997), if the concentrations of salivary cotinine were found to be below the limit of

2063656432

Source: https://www.industrydocuments.ucsf.edu/docs/qfpk0000

quantification (LOQ) or limit of detection (LLD), the values were empirically set equal to one-half of the LLD or LOQ. Depending on a number of factors and the criteria used to establish the LLD or LOQ, this means that in some cases up 60% of the data was set to 0.5 ng/mL for salivary cotinine. With so few data points, a linear regression with an intercept near 0.5 ng/mL appears reasonable. However, one does not know what the values may be if they are below the LLD or LOQ. The analytical method used by Pirkle et al. (Pirkle et al., 1996; Bernert et al., 1997) has a reported detection limit of 50 ng/L (0.05 ng/mL). Based on estimates calculated from data shown in Figure 2 in Pirkle et al. (1996), approximately 50 % of the data for nonsmokers is found in the region below 0.5 ng/mL, further bringing into question the data treatment used in exposure studies.

Other Factors that Affect Cotinine Concentration in Biological Fluids

From the data discussed so far, it appears that salivary and serum cotinine concentrations may be correlated to exposure to nicotine in ETS for nonsmokers. Almost all exposure studies have been conducted with mature adults >17 years of age. However, a number of studies have suggested that cotinine levels for a given exposure are higher in children and blacks (Zuccaro et al., 1997; Wagenknecht et al., 1993; Pattishall et al., 1985). In one of the few studies to attempt to use multivariate methods of analysis of exposure data, Pirkle et al. (1996) performed multiple regression of log serum cotinine concentration versus such parameters as age, ethnic background, number of smokers in the house, size of household, number of rooms in the house, etc. Dietary intake was considered in the form of consumption of bell peppers and found to be of

2063656433

Source: https://www.industrydocuments.ucsf.edu/docs/qfpk0000

marginal statistical significance ($0.001<p<0.05$) in children 4-11 years of age, but not significant in adults. The regression coefficients that were obtained had a wide range of statistical reliability.

As an illustrative example, **Figure 2** shows a plot of serum cotinine concentrations calculated using regression coefficients from Pirkle versus age for white and black children living in a 5-room house, with 3 members in the household, with 1 smoker, and after consuming 20 g of bell pepper. There are two observations from **Figure 2**: all other factors being equal, young children show cotinine levels higher than adolescents, and black children have higher cotinine levels than whites. The statistical significance of many of the regression coefficients was marginal, but a trend is suggested.

In a 1984 report, Greenberg et al. determined urinary and salivary cotinine levels in infants and reported urinary cotinine to be the most reliable indicator of exposure. Median levels of cotinine in urine were 351 ng and 4 ng of cotinine/mg creatinine for ETS exposed and not exposed, respectively. From these data, Van Vunakis et al. (1987) have estimated that on a creatinine basis, urinary cotinine levels in infants are approximately 60 times greater than those found in adult males. Based on urine volume, the concentration of cotinine in the urine of infants is 8 and 1.6 times as much as the ETS-exposed and highly exposed groups, respectively. Weaver et al. (1996) determined the urinary cotinine levels of 79 inner-city children and found a mean of 54.7 ± 45.6 ng/mL with a range of 1-244 ng/mL.

2063656434

Source: https://www.industrydocuments.ucsf.edu/docs/qfpk0000

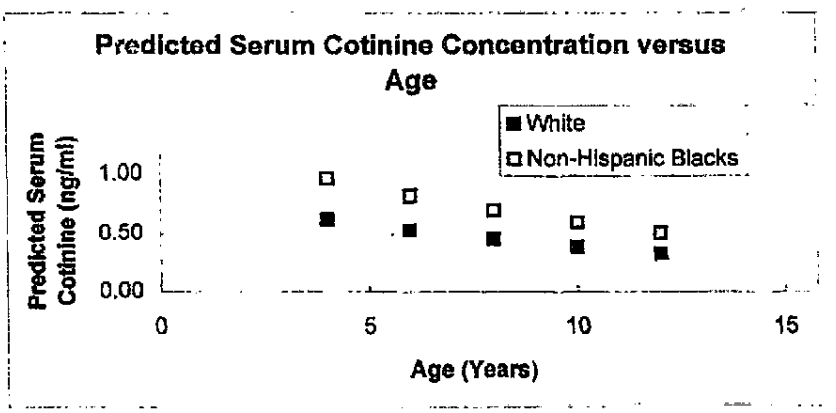

Figure 2. Use of Multivariate Analysis of NHANES III" Data to Illustrate the Effect of Age and Ethnicity
(NHANES III = Third National Health and Nutrition Examination Survey)

2063656435

Source: https://www.industrydocuments.ucsf.edu/docs/qfpk0000

Clearly, the age and ethnic background of young children must be taken into account when the cotinine levels of this group are used to infer levels of exposure to ETS. If this factor is not taken into consideration, cotinine levels found in exposure studies can be misinterpreted as excessive exposure.

Conclusions

Cotinine is not a perfect biomarker for nicotine exposure. Issues such as interpersonal variation in metabolism, non-tobacco sources of nicotine, and how well nicotine represents ETS exposure plague all biomarkers. For the present, cotinine appears to be the most reliable biomarker for estimating day-to-day exposure to tobacco smoke provided that it is applied only to mean (or median) values of large numbers of subjects.

There is much room to improve upon the use of nicotine metabolites as biomarkers for nicotine intake. Cotinine in serum and saliva appear to be equally advantageous over the use of urine samples; principally because of the limited number of analytically significant metabolites in these media. This is especially true if RIA methods are used, because of the potential for cross-reactivity of metabolites other than cotinine. Further, because cotinine represents 10-15% of the total nicotine metabolite in urine, variation in the larger concentrations of the other metabolites could have significant impact on the use of cotinine only in relating the result to nicotine intake.

2063656436

Source: https://www.industrydocuments.ucsf.edu/docs/qfpk0000

A viable analytical method that has a detection limit much below that of RIA and gas chromatography is needed. The method must be rugged and capable of processing large numbers of samples economically. Such a method has been developed for cotinine in serum based on liquid chromatography combined with atmospheric pressure ionization tandem mass spectrometry (LC/API/MS/MS) (Wagenknecht et al., 1993). Work is underway to apply the method to saliva samples. Initial capital outlay is high, but the ruggedness and throughput capabilities of the method are excellent.

However, saliva and serum have their own limitation as a sample matrix. Only intensive or concentration measurements may be made. To obtain data on the equivalent of nicotine eliminated over a period of time, total nicotine metabolite excretion by collecting urine samples has a number of advantages. **Table 2** shows that approximately 90% of the total nicotine metabolite in urine could potentially be estimated by that approach. Therefore, a method using enzymatic hydrolysis of the glucuronide conjugates of nicotine and its metabolites in urine, with the subsequent determination of nicotine, cotinine and *trans*-3-hydroxycotinine is required. This method should have a detection limit to permit quantitative data to be obtained for subjects with the lowest level of nicotine exposure. It is likely that modification of existing procedures coupled with LC/API/MS/MS could meet these needs.

2063656437

Source: https://www.industrydocuments.ucsf.edu/docs/qfpk0000

What Is Philip Morris Doing About Cotinine

Until an even more appropriate biomarker for nicotine intake is found, cotinine is currently the one of choice. The issue is not cotinine *per se*, but the limitations and misinterpretations associated with measurements and data. Several factors need to be considered. Some of these are:

1. Which biological fluid is overall the most appropriate as the measurement medium?

2. What are the recommended procedures and protocols for the acquisition, transportation, preservation and storage of these samples?

3. Which nicotine metabolites should one measure to obtain improved and appropriate accuracy in the estimation of nicotine intake?

4. What analytical method is best suited to the task?

5. How should data be analyzed?

6. How can the proliferation of expedient but questionable analytical practices be contained?

2063656438

Source: https://www.industrydocuments.ucsf.edu/docs/qfpk0000

A number of these issues are being addressed directly or indirectly by Philip Morris-supported programs.

1. *Which biological fluid is overall the most appropriate as the measurement medium?*

Each of the principal biological fluids used for biomarker measurements, serum, saliva and urine, has advantages and disadvantages as described above. The analytical methods that may be applicable also have a range of strong and weak points. In 1986, a group of experts met to discuss these issues in detail (Watts et al., 1990). This group concluded that "estimation based on urinary cotinine excretion would be less reliable than estimation based on plasma or salivary levels." They also found that "good correlations were reported between saliva and blood for results from the same subject," as found by others (Curvall et al., 1989). The number of nicotine metabolites in urine, the interpersonal variation, and the issues of using the CCR method that result from creatinine concentration variation, all counter the relative convenience of collecting urine samples.

Following the recommendations given above, Philip Morris is participating in a program to carefully investigate the use of saliva as the sample medium of choice. Although urine has considerable interest from a pharmacological perspective, as a sample medium used strictly to estimate nicotine exposure, either serum or saliva appears to have the advantage. Work is underway to establish appropriate sampling protocols and analytical methodologies for

2063656439

Source: https://www.industrydocuments.ucsf.edu/docs/qfpk0000

saliva. An excellent method for cotinine determinations in serum was recently published (Bernert et al., 1997).

2. *What are the recommended procedures and protocols for the acquisition, transportation, preservation and storage of these samples?*

A number of recommendations for sample acquisition and handling for saliva have been reported. The subject is too detailed to be reviewed here. Philip Morris is participating in a study to evaluate a number of factors in the subject preparation, sample collection, sample preservation, sample transportation and storage for saliva. A method and procedures document is planned.

3. *Which nicotine metabolites should one measure to obtain improved and appropriate accuracy in the estimation of nicotine intake?*

For a variety of reasons, the recommendations reported above (Watts et al., 1990) have not been adopted by many researchers who conduct epidemiological investigations, particularly with respect to the use of urine as the sample medium. If nicotine metabolites are to be used in urine, improved analytical methods are required for acceptable results because of the number and variation in the nicotine metabolites. Philip Morris is conducting a statistical analysis of existing data to address this issue. The outcome of this analysis will be an

2063656440

Source: https://www.industrydocuments.ucsf.edu/docs/qfpk0000

opportunity to define the analytical needs to minimize variations resulting from the complexities of a mixture of metabolites.

4. *What analytical method is best suited to the task?*

Philip Morris is participating in the development of an analytical method for cotinine and possibly *trans*-3-hydroxycotinine in saliva. For cotinine at least, the lower limit of quantification (LOQ) is expected to be approximately 0.05 ng/mL using conservative statistical policies. This may be compared with the current approximately 0.5-1.0 ng/mL using RIA methods. The method will have relatively high initial capital costs, but is capable of a throughput of over 100 samples per day. Under consideration is the participation in the development of an alternative method for urine that will involve enzymatic hydrolysis of the glucuronide conjugates of nicotine, cotinine and *trans*-3-hydroxycotinine with the subsequent determination of these three metabolites.

5. *How should data be analyzed?*

There are two issues with data analysis relative to cotinine that are present in the literature.

a.) In exposure studies, cotinine levels below the limit of quantification (LOQ) of the analytical method used are empirically set equal to 50% of the LOQ. This has the negative impact that regardless of how much ETS exposure is reduced, a finite

2063656441

Source: https://www.industrydocuments.ucsf.edu/docs/qfpk0000

cotinine level is guaranteed. This can result in a misuse of values below an LOQ. As ETS exposure is reduced, new analytical methods are required to demonstrate reduction in nicotine intake.

b.) Smoker-nonsmoker discrimination is based on the use of a point value "cut-off" approach. This assumes that anyone below a set value of cotinine level is a nonsmoker, and anyone above that value is a smoker. This is not consistent with biological diversity. New methods of distributive data analysis that permit incorporation of a variety of factors to predict the probability of a single result are needed. Philip Morris is participating in the evaluation of such methods of data analysis.

6. *How can the proliferation of expedient but questionable analytical practices be contained?*

For a variety of reasons, often commercial, new quick and easy methods for the detection and alleged quantification of cotinine or other biomarker for nicotine appear in the literature, in patents or in the marketplace. These devices offer quick, simple and near-patient determinations that may be used for various purposes, such as establishment of smoking status for insurance applications. The complexities described above all but preclude successful use of such simple, nonselective "dip-stick" tests. One such device is known as NicCheck™ on the market and is FDA approved for physician use for detection of smokers.

2063656442

Source: https://www.industrydocuments.ucsf.edu/docs/qfpk0000

Philip Morris is participating in an investigation of this device to ascertain its reliability, especially against potential false positive results.

2063656443

Source: https://www.industrydocuments.ucsf.edu/docs/qfpk0000

Appendices

The following papers are appended to this section:

Schepers, G., Demetriou, D., Rustemeier, K., Voncken, P., and Diehl, B., Nicotine phase 2 metabolites in human urine -- structure of metabolically formed *trans*-3'-hydroxycotinine glucuronide, *Med. Sci Res.* 20: 863-865 (1992). **[Tab A]**

Schepers, G., Rustemeier, K., Walk, R.A., and Hackenberg, U., Metabolism of S-nicotine in noninduced and Aroclor-induced rats, *Eur. J. Drug Metab. and Pharmacokin.* 18: 187-197 (1993). **[Tab B]**

Schepers, G., and Walk, R.A., Cotinine determination by immunoassays may be influenced by other nicotine metabolites, *Arch. Toxicol.* 62: 395-397 (1988). **[Tab C]**

2063656444

Source: https://www.industrydocuments.ucsf.edu/docs/qfpk0000

References

Andersson, G., Vala, E.D., Curvall, M., The influence of cigarette consumption and smoking machine yields of tar an nicotine on the nicotine uptake and oral mucosal lesions in smokers. *J Oral Pathol Med* 26:117-123 (1997).

Benner, C.L., Bayona, J.M., Caka, F.M., et al., Chemical composition of environmental tobacco smoke. 2. Particulate-phase compounds. *Environ Sci Technol* 23:688-699 (1989).

Benowitz, N.L., Cotinine as a biomarker of environmental tobacco smoke exposure, *Epidemiol. Rev.* 18:188-204 (1996).

Benowitz, N.L., Zevin, S., Jacob, P., *Br. J. Clin Pharmacol* 43:259-267 (1997).

Bernert, J.T., Wazman, E.T., Pirkle, J.L., Sosnoff, C.S., Akins, J.R., et al., Development and validation of sensitive method for determination of serum cotinine in smokers and nonsmokers by liquid chromatography/atmospheric pressure ionization tandem mass spectrometry. *Clin Chem* 43(12): 2281-2291 (1997).

Byrd, G.D., Robinson, J.H., Caldwell, W.S., deBethizy, J.D., Nicotine uptake and metabolism in smokers. Paper presented at the 48th Tobacco Chemists' Research Conference, Greensboro, NC, September 25-28, 1994.

Castro, A., Monji, N., Dietary nicotine and its significance in studies on tobacco smoking. *Biochem Arch* 2: 91-97 (1986).

Curvall, M., Elwin, C.E., Kazemi-Vala, E., et al., The pharmacokinetics of cotinine in plasma and saliva from non-smoking healthy volunteers. *Eur J Clin Pharmacol* 38:281-287 (1990).

Curvall, M.,Vala, E.D., Englund, G., Enzell, C.R., Urinary excretion of nicotine and its major metabolites. Paper presented at the 43rd Tobacco Chemists' Research Conference, Richmond, VA, September 29-October 3, 1989.

Davis, R.A., Stiles, M.F., deBethizy, J.D., Reynolds, J.H., Dietary nicotine: a source of urinary cotinine. *Fd Chem Toxic* 29: 821-827 (1991).

Domino, E.F., Nontobacco sources of cotinine in the urine of nonsmokers. *Clin Pharmacol Ther* 57: 479 (1995).

Domino, E.F., Horbach, E., Demana, T., Relevance of nicotine content of common vegetables to the identification of passive tobacco smokers. *Med. Sci. Res.* 21: 571-572 (1993).

2063656445

Source: https://www.industrydocuments.ucsf.edu/docs/qfpk0000

Greenberg, R.A., Haley, N.J., Etzel, R.A., Loda, F.A., Measuring the exposure of infants to tobacco smoke. Nicotine and cotinine in urine and saliva. *New Engl J Med* 310: 1075-1078 (1984).

Greenberg, R.S., "Prospective studies" In: *Encyclopedia of Statistical Sciences*. V. 7, pp. 315-319, S. Kotz and N.L. Johnson, Eds., John Wiley & Sons, NY (1986).

Jarvis, M., Tunstall-Pedoe, H., Feyerabend, C., et al., Biochemical markers of smoke absorption and self-reported exposure to passive smiling. *J Epidemiol Community Health* 38:335-339 (1984).

Jenkins, R.A., Palausky, A., Counts, R.W., et al., Exposure to environmental tobacco smoke in sixteen cities in the United States as determined by personal breathing zone air sampling. *J Expo Anal Environ Epidemiol.* 6: 473-502 (1996).

Kemmeren, J.M., van Poppel, G., Verhoef, P., Jarvis, M.J., Plasma cotinine: stability in smokers and validation of self-reported smoke exposure in nonsmokers. *Environ. Res.* 66:235-243 (1994).

Kyerematen, G.A., Taylor, L.H., deBethizy, J.D., Vessell, E.S., Radiometric-high performance liquid chromatography assay for nicotine and twelve of its metabolites. *J Chrom Biomed Appl* 419:191-203 (1987).

Langone, J.J., Gjika, H.B., Van Vunakis H., Nicotine and its metabolites. Radioimmunoassays for nicotine and cotinine. *Biochemistry* 12: 5025-503 (1973).

National Research Council, Committee on Passive Smoking, Board of Environmental Studies and Toxicology, *Environmental tobacco smoke: measuring exposures and assessing health effects*. Washington D.C.: National Academy Press (1986).

Neurath, G.B., Pein, F.G., Gas chromatographic determination of trans-3'-hydroxycotinine, a major metabolite of nicotine in smokers. *J Chrom Biomed Appl* 15: 400-406 (1987).

Pattishall, E.N., Strope, G.L., Etzel, R.A., Helms, R.E., Haley, N.J., Denny, F.W., Serum cotinine as a measure of tobacco smoke exposure in children. *AJDC* 139:1101-1104 (1985).

Phillips, K., Bentley, M.C., Howard, D.A., Alvan, G., Assessment of air quality in Stockholm by personal monitoring of nonsmokers for respirable suspended particles and environmental tobacco smoke. *Scan J Work Environ Health* 22 (Suppl. 1)1:1-24 (1996).

Phillips, K., Bentley, M.C., Howard, D.A., Alvan, G., and Huici, A., Assessment of air quality in Barcelona by personal monitoring of nonsmokers for respirable suspended particles and environmental tobacco smoke. *Environ Inter* 23:173-196 (1997).

2063656446

Source: https://www.industrydocuments.ucsf.edu/docs/qfpk0000

Pirkle, J.L., Flegal, K.M., Bernert, J.T., et al., Exposure of the US population to environmental tobacco smoke: The Third National Health and Nutrition Examination Survey. *JAMA* 275:1233-1240 (1996).

Riboli, E., Haley, N.J., Tredaniel, J., Saracci, R., Preston-Martin, S., Trichopoulos, D., Misclassification of smoking status among women in relation to exposure to environmental tobacco smoke. *Eur Respir J* 8:285-290 (1995).

Saracci, R., Riboli, E., Passive smoking and lung cancer. current evidence and on-going studies at the International Agency for Research on Cancer, *Mutat Res.* 222:117-127 (1989).

Sheen, S.J., Detection of nicotine in foods and plant materials. *J. Food Sci.* 53:1572-1573 (1988).

Statements attributed to D. Trichopoulos, E. Kakalamani and A. Linou in Greek Newspaper "Ta Nea," April 30, 1997.

Turner, D.M., *Biochem J* 115: 889 (1969).

Van Vunakis, H., Gjika, H.B., Langone, J.J., In: *Environmental Carcinogens: Methods of Analysis and Exposure Measurement, V. 9 -- Passive Smoking*, O'Niel, I.K., Brunneman, K.D., Dodet, B., Hoffmann, D. (Eds.) International Agency for Research on Cancer, Lyon, p. 322 (1987).

Wagenknecht, L.D., Manolio, T.A., Sidney, S., et al., Environmental tobacco smoke exposure as determined by cotinine in black and white young adults: The CARDIA Study. *Environ Res.* 63:39-46 (1993).

Watts, R.R., Langone, J.J., Knight, G.J., Lewtas, J., Cotinine analytical workshop report: onsideration of analytical methods for determining cotinine in human body fluids as a measure of passive exposure to tobacco smoke. *Environ Health Perspect* 84: 173-182 (1990).

Weaver, V.M., Davoli, C.T., Murphy, S.E., Sunyer, et al., Environmental Tobacco Smoke Exposure in Inner-City Children. *Can Epidemiol Biomarkers & Prev.* 5:135-137 (1996).

Zuccaro, P., Pichini, S., Altiere, I., Rosa, M., Pellegrini, M., Pacifici, R., *Clin Chem* 43:180-181 (1997).

2063656447

Source: https://www.industrydocuments.ucsf.edu/docs/qfpk0000